Will PAXgene substitute formalin? A morphological and molecular comparative study using a new fixative system

Belloni, Benedetta ; Lambertini, Chiara ; Nuciforo, Paolo ; Phillips, Jay ; Bruening, Eric ; Wong, Stephane ; Dummer, Reinhard

Abstract: Formalin fixation and paraffin embedding present the standard procedures for conserving clinical tissues for histological analysis. However, molecular analysis is impaired by the cross linking properties of formalin. The PAXgene tissue system (PreAnalytix, Switzerland) is a new formalin-free tissue collection device. AIMS: In this study we aimed to evaluate this new tissue preservation technique in comparison with formalin fixation and fresh frozen tissue samples. METHODS: 12 melanoma biopsy samples were divided and fixed simultaneously with formalin, PAXgene or fresh frozen in liquid nitrogen and analysed with regard to morphology, immunohistochemistry, DNA and RNA content and quality. Markers of melanocytic differentiation and tumour cell proliferation were used. RESULTS: Morphology was well preserved in PAXPE samples. However, 5 out of 11 immunohistochemical markers showed significantly lower overall staining and staining intensity with PAXPE tissues in comparison with formalin-fixed, paraffin-embedded (FFPE). Increasing membrane permeability through adding a detergent did proportionally increase staining intensity in PAXPE samples. Amplification of different mRNA amplicons showed a direct relationship with the size of the amplicon with greater template integrity observed in PAXPE samples. Sequencing and mutational analysis of DNA samples were comparable for all the different fixation methods, while the level of DNA fragmentation seemed to be lower in PAXPE compared with FFPE tissues. CONCLUSIONS: The switch from formalin to PAXgene fixation would require a re-evaluation of immunohistochemical markers and staining procedures originally developed for FFPE tissues. Our data demonstrate that PAXPE fixation offers some advantages concerning molecular analysis. However, these advantages would not justify substituting formalin fixation in any routine pathology laboratory.

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Will PAXgene substitute formalin? A morphological and molecular comparative study using a new fixative system

Benedetta Belloni,[1] Chiara Lambertini,[2] Paolo Nuciforo,[2] Jay Phillips,[3] Eric Bruening,[3] Stephane Wong,[3] Reinhard Dummer[1]

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[1]Department of Dermatology, University Hospital of Zurich, Zurich, Switzerland
[2]Oncology Translational Medicine, Novartis, Basel, Switzerland
[3]Molecular MD, Oregon, USA

Correspondence to
Professor Dr Reinhard Dummer, Department of Dermatology, University Hospital Zurich, Zurich, 8091, Switzerland; Reinhard.Dummer@usz.ch

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ABSTRACT

Formalin fixation and paraffin embedding present the standard procedures for conserving clinical tissues for histological analysis. However, molecular analysis is impaired by the cross linking properties of formalin. The PAXgene tissue system (PreAnalytix, Switzerland) is a new formalin-free tissue collection device.

Aims In this study we aimed to evaluate this new tissue preservation technique in comparison with formalin fixation and fresh frozen tissue samples.

Methods 12 melanoma biopsy samples were divided and fixed simultaneously with formalin, PAXgene or fresh frozen in liquid nitrogen and analysed with regard to morphology, immunohistochemistry, DNA and RNA content and quality. Markers of melanocytic differentiation and tumour cell proliferation were used.

Results Morphology was well preserved in PAXPE samples. However, 5 out of 11 immunohistochemical markers showed significantly lower overall staining and staining intensity with PAXPE tissues in comparison with formalin-fixed, paraffin-embedded (FFPE). Increasing membrane permeability through adding a detergent did proportionally increase staining intensity in PAXPE samples. Amplification of different mRNA amplicons showed a direct relationship with the size of the amplicon with greater template integrity observed in PAXPE samples. Sequencing and mutational analysis of DNA samples were comparable for all the different fixation methods, while the level of DNA fragmentation seemed to be lower in PAXPE compared with FFPE tissues.

Conclusions The switch from formalin to PAXgene fixation would require a re-evaluation of immunohistochemical markers and staining procedures originally developed for FFPE tissues. Our data demonstrate that PAXPE fixation offers some advantages concerning molecular analysis. However, these advantages would not justify substituting formalin fixation in any routine pathology laboratory.

INTRODUCTION

Formalin fixation and paraffin embedding present the standard procedures for conserving clinical tissues, enabling long-term storage of human samples. In combination with antigen retrieval (AR) methods it enables high-quality immunohistochemical analysis.[1] However, the cross linking properties of formalin cause fragmentation of nucleic acids, impairing the quality of extracted DNA and RNA[2–4] The gold-standard for molecular analysis remains fresh-frozen tissue, which is not perfect for accurate histological and morphological studies. Furthermore, the long-time storage is complicated and cost-intensive.

Thus, there is a need for a fixative enabling high quality morphological and immunohistochemical, and molecular analysis. Other important issues in tissue preparation include fixation times, tissue shrinkage and hardening, degradation of phosphoepitopes, handling and transport of carcinogenic and/or flammable fixatives. There are alternative, alcohol based, fixatives to formalin in the market, none of which have replaced formalin in routine analysis up to date.[5–10]

The PAXgene tissue system (PreAnalytix, Switzerland) consists of a commercially available, easy to handle, dual-chamber tissue collection device. According to the manufacturer's product description, the containers are prefilled with 2 reagents (PAXgene Tissue Fixation Reagent and PAXgene Tissue Stabilisation Reagent) consisting of methanol and acetic acid as well as a soluble organic compound for fixation and a mixture of different alcohols including ethanol for stabilisation. The reagents are designed to preserve morphology, protein content and, moreover, nucleic acid integrity in fixed tissues.

In this study we aimed to evaluate this new tissue preservation technique in comparison with formalin fixation and fresh frozen tissue samples. The preservation of morphology and the performance in immunohistochemistry (IHC), and nucleic acid based analysis were analysed.

MATERIAL AND METHODS

Tissue samples

12 tissue samples of primary or metastatic malignant melanoma (two primary melanomas, three lymph node metastases, and seven skin/subcutaneous metastases) were obtained from patients undergoing surgery at the Dermatology Department, University hospital Zurich (table 1). Specimens were divided into three samples immediately after surgery and were either optimal cutting temperature compound (OCT)-embedded and snap-frozen in liquid nitrogen, or PAXgene-fixed (2–4 h fixation at 4°C) or formalin-fixed (24h fixation at RT). Samples for PAXgene-fixation had a maximum size of 4×10×10 mm as recommended by the manufacturer. The PAXgene-fixed samples were transferred into PAXgene Tissue Stabilisation Reagent for at least 24 h at 4°C.

After dehydration of PAXgene-fixed and formalin-fixed tissues, the samples were embedded either in low melting paraffin for the PAX-gene-fixed tissues or

Table 1 Patient material information

Patient ID	Patient's information		Biopsy information		
	Sex	Age (years)	Tissue type	Region	Primary tumour/ Metastasis
PAX01	F	64	Lymphnode	Inguinal	Metastasis
PAX02	M	75	Skin	Back	Metastasis
PAX03	M	68	Skin	Shoulder	Metastasis
PAX04	M	79	Skin	Foot	Primary tumour
PAX05	M	72	Skin	Back	Metastasis
PAX06	M	68	Skin	Knee	Metastasis
PAX07	F	61	Skin	Upper leg	Metastasis
PAX08	F	74	Subcutaneous	Neck	Metastasis
PAX09	M	65	Skin	Front	Primary tumour
PAX10	M	84	Lymphnode	Inguinal	Metastasis
PAX11	M	49	Skin	Back	Metastasis
PAX12	F	76	Lymphnode	Inguinal	Metastasis

in normal paraffin for the formalin fixed. The protocol consists of stepwise dehydration in 70%, 96%, 100% ethanol, followed by xylene. PAXgene-fixed and formalin-fixed, paraffin-embedded (FFPE) tissue blocks were kept in the dark at 4°C or at room temperature, respectively.

All patients gave informed consent and the study was approved by the Ethics Committee of Zurich, Switzerland (reference number EK 800).

HISTOCHEMISTRY AND IHC ANALYSIS

H&E staining was performed according to standard procedures, and the sections were histologically verified by two board-certified pathologists (PN, RD). Morphology assessment included overall morphology and nuclear, cytoplasmic and membrane details in PAXPE and FFPE tissues.

For IHC analysis on tissues, 3 μm thick paraffin sections were prepared using a rotating microtome (Microm, HM 355 S). Sections were deparaffinised in xylene followed by hydration in a graded series of ethanol (100%, 95% and 70% ethanol) and water. Sections underwent AR pre-treatment following the specific protocol for each antibody used (see online supplementary table 1). IHC experiments were performed using either the DAKO autostainer plus (Glostrup, Denmark) or the Ventana Discovery XT automatic platform (Ventana Medical System, Tucson, Arizona, USA). To visualise immunoreactivity, anti-mouse-horseradish peroxidase or streptavidine-alkaline-phosphatase was added to the slides followed by either 3,3'-Diaminobenzidin (DAB) solution or fast RED solution. Sections were counterstained and dehydrated in a graded series of alcohol (70%, 95% and 100%), incubated in xylene and mounted.

Antibodies against MelanA, Tyrosinase (Novocastra, Newcastle, UK), p16 (BD, Transduction Laboratories, San Jose, USA), Mage A4 (generous gift by Prof Giulio C. Spagnoli, Department of Surgery, University Hospital Basel, Switzerland), b-Raf (Abcam, Cambridge, UK), cyclinD1 (CCND1) (Epitomics/ Biocare, Concord/ Burlingame, California, USA), c-Kit (Dako, Glostrup, Denmark), Ki67 (Dako, Glostrup, Denmark), microphthalmia-associated transcription factor (MITF) (Dako, Glostrup, Denmark), p53 (DAKO, Glostrup, Denmark), S100 (Ventana, Tuscon, Arizona, USA) were used as described in the online supplementary table 1.

For Ki67 and p53, varying antibody concentrations were applied (1 : 100, 1 : 200 and 1 : 500, 1 : 1000, respectively). Different AR solutions administered for anti-CCND1 and anti-p53 antibodies were pH6, pH9 and noAR (noAR). For CCND1, MITF, and p53, staining and permeabilisation of tissue samples was achieved by adding NP-40 0.4% solution (diluted in PBS) for 4 min or 30 min at room temperature before the blocking solution.

Quality of staining and scoring were assessed using an optic microscope (Zeiss Axio Imager A1 and Axiolab). Levels of immunoreactivity in the melanoma samples were evaluated by pathologists using the histo-score (H-score), which calculates the sum of the percentage of cells stained (0–100) and the staining intensity (0–3). For example: a specimen with 10% of cells staining 3+ (strong), 20% of cells staining 2+ (moderate), 30% of cells staining 1+ (low) and 40% of cells unstained would have a complete H-score of $(3\times10)+(2\times20)+(1\times30)=100$. The resulting score ranges from 0 (no staining in the tumour) to 300 (diffuse intense staining of the tumour).[11 12]

STATISTICAL ANALYSIS

Data were analysed by unpaired student t-test for independence. Two groups (FFPE and PAXPE) were compared. Differences were considered significant when p values were ≤ 0.05.

NUCLEIC ACID EXTRACTIONS

Nucleic acids for each of the three tissue preservation methods were extracted from 12 patient specimens. Total RNA was isolated from each specimen with preservation specific methods using the manufacturer's recommended protocols. PAXPE RNA was prepared using the PAXgene Tissue RNA Kit (PreAnalytiX GmbH, Switzerland), and FFPE RNA was prepared using BiOstic FFPE Tissue RNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, California, USA.). Fresh frozen OCT embedded tissue was washed briefly with 4°C phosphate buffered saline and RNA isolated by Trizol extraction (LifeTechnologies, Carlsbad, California, USA). DNA was extracted from additional sections from PAXPE and FFPE fixation methods using the BiOstic Tissue DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, California, USA) using the manufacturer's recommended protocol. Extracted nucleic acids were quantified by absorbance (Nanodrop, Thermo Scientific Inc., Wilmington, Delaware, USA) and evaluated for impurities by 260/280 and 260/230 ratios for residual protein and chemical extraction contaminants respectively.

RNA AMPLIFICATION QUALITY: REVERSE TRANSCRIPTION PCR ASSAYS (QRT-PCR)

qRT-PCR assays were designed to expose subtle differences in the functional copy number of mRNA expressed in the sample set. The RNA template quality as a function of preservation method is reflected in the amplifiable target copy number. Taqman assays were designed with nested amplification primers (Integrated DNA technologies Coralville Iowa, USA), and a universal taqman MGB probe (Applied Biosystems). Reactions were performed using the Applied Biosystems (ABI) 7500 Fast Real-Time PCR System. Resulting crossing threshold (Ct) data were analysed and quantified with the 7500 System SDS Software, V.1.3.1 (ABI), utilising the second derivative maximum method.

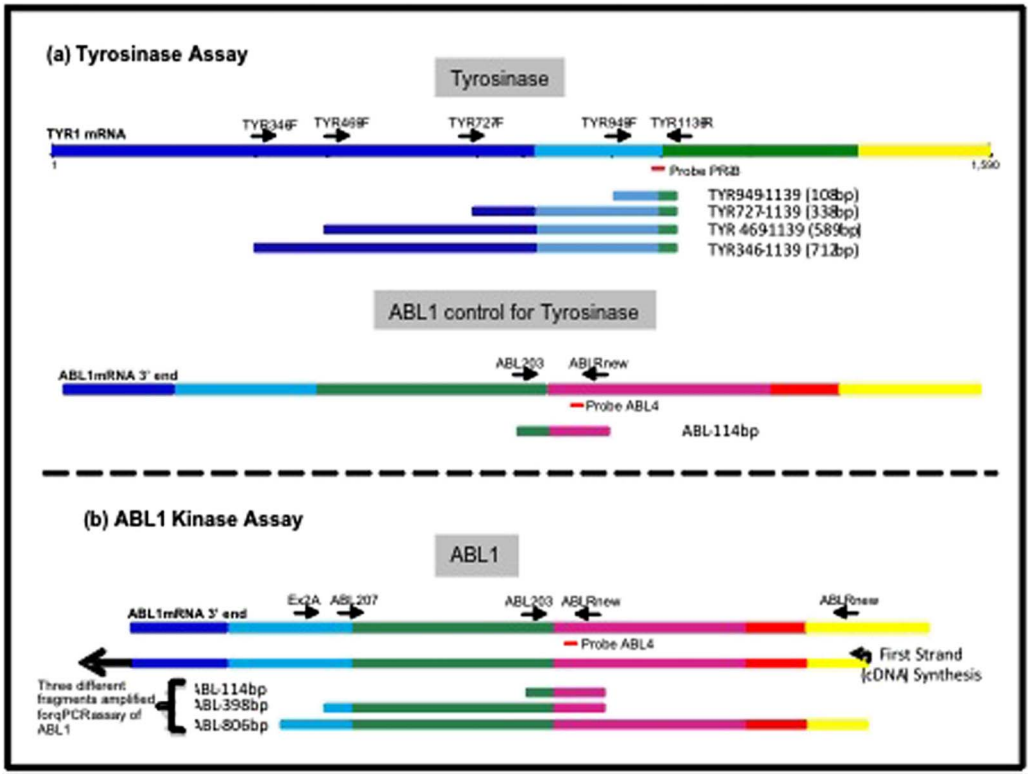

Figure 1 RNA Amplification Quality RT-PCR Assays. Relative position of PCR amplification primers and detection probes for each RT-PCR assay is represented above the respective mRNA targets. The different coloured segments of the mRNA represent the different exons of the sequence. Figures below the mRNA represent the amplification products for each assay, and show the relative position on the mRNA target. This figure is only reproduced in colour in the online version.

ABL1 kinase assay

ABL1 Kinase (ABL1, (NM_005157) mRNA was the target for each of three assays designed to evaluate fixation method specific preservation of a steady state transcript. ABL1 comparison of nucleic acid quality was performed using 2-step RT-PCR assay chemistry designed to amplify nested fragments of different lengths from the same target mRNA in separate, parallel, reactions (figure 1B). Amplification primers for each of the different ABL1 RT-PCR assays are outlined in table 2A.

First strand cDNA synthesis of ABL1 from total RNA was generated using the target specific ABL r2 primer for each of the assays using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, California, USA) following the manufacturers recommended concentrations for all reagents, and 500 ng total RNA as template in 50 µl total reaction volume. RT-PCR amplification and detection of ABL1 was performed in MicroAmp Optical 96-well Reaction Plates (ABI). The final reaction volume of 25 µl using AmpliTaq Gold DNA Polymerase with GeneAmp 10X PCR Gold Buffer (Life Technologies Corporation, Carlsbad, California, USA), using 4.5 mM $MgSO_4$, 50 mM 6-carboxy-X-Rhodamine (ROX) reference dye and 5 µl of the first strand synthesis reaction as template. RT-PCR reactions included 800 µM of each assay specific amplification primer (forward and reverse) and 400 µM TaqMan-MGB 6-Carboxy-fluorescine (FAM) labelled probe ABL4. Each reaction was performed in duplicate. Thermal cycling for detection of ABL targets in the 2-step RT-PCR reaction used an initial denaturing incubation (95°C, 10 min) followed by 50 cycles of PCR (20 s at 95°C, 20 s at 57°C, 90 s at 72°C).

Tyrosinase qRT-PCR assay

Tyrosinase (TYR, NM_000372) mRNA was evaluated and normalised to the stably expressed control gene ABL1 (ABL1, (NM_005157). Nested fragments of increasing lengths for the

Table 2 RNA amplification quality assay oligonucleotides

Oligonucleotide ID	Oligo Function:	Primer Sequence (5′ to 3′):
(A) ABL Kinase (ABL1) RNA amplification quality assay primers		
ABL203	Forward PCR Primer	AGGGAGGGTGTACCATTACAGGAT
ABL207	Forward PCR Primer	GCCAGTGGAGATAACACTCTAAGCA
ABL Ex2A	Forward PCR Primer	ACCCCAACCTTTTCGTTGC
ABL Rnew	Reverse PCR Primer	GCCACCGTTGAATGATGATGAACC
ABL r1	cDNA Synthesis Primer	GCACTC CCTCAGGTAGTCCA
ABL4	Detection Probe	CCTGGCCGAGTTGGTT
(B) Tyrosinase (TYR1) RNA amplification quality assay primers		
TYR346-F	Forward PCR Primer	CGACTCTTGGTGAGAAGAAAC
TYR467-F	Forward PCR Primer	GGCCAAATGAAAAATGGATCA
TYR727-F	Forward PCR Primer	GCAGAAAAGTGTGACATTTGCAC
TYR949-F	Forward PCR Primer	GATGTAGAATTTTGCCTGAGTT
TYR1139-R	Reverse PCR Primer	CAGTAAGTGGACTAGCAAATCCTTC
PR3-3	Detection Probe	CAGCTTTAGAAATACACT
ABL Rnew	Reverse PCR Primer	GCCACCGTTGAATGATGATGAACC
ABL r1	cDNA Synthesis Primer	GCACTC CCTCAGGTAGTCCA
ABL4	Detection Probe	CCTGGCCGAGTTGGTT

Oligonucleotides for each of the RNA amplification quality assays are listed in separate tables. Detection probes are TaqMan MGB FAM labelled oligonucleotides.

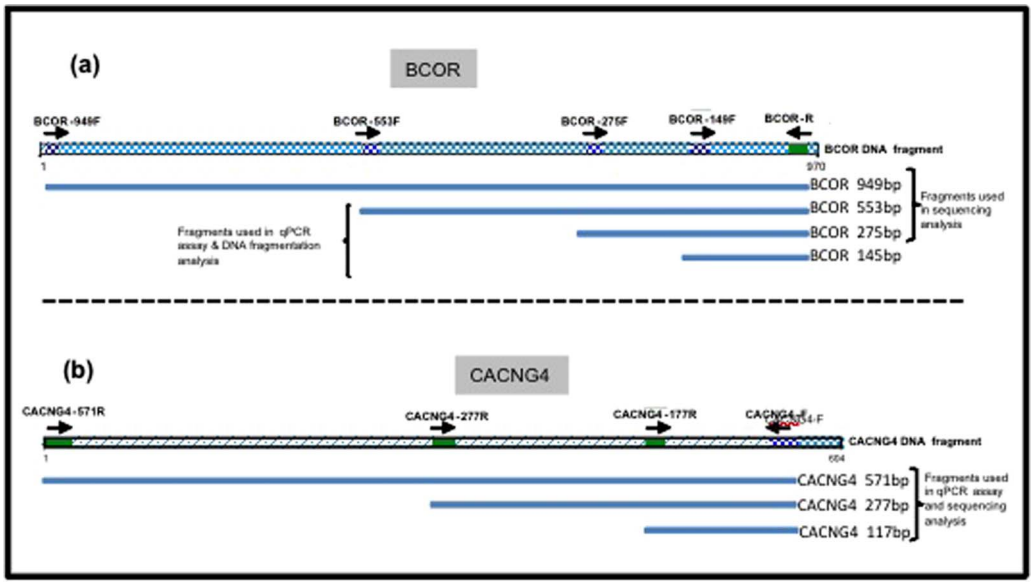

Figure 2 DNA quality RT-PCR and Sequencing Assays. Fragments of genomic DNA targets are represented with relative positions of amplification primers (black arrows) above the DNA fragment. The different amplicons for each assay are represented below the DNA fragment, to show the position of the different amplicons relative to the DNA target as well as their relative position and overlap to each other. This figure is only reproduced in colour in the online version.

target mRNA were evaluated in separate parallel reactions. Cycling conditions were optimised to attain similar qRT-PCR amplification efficiencies for each of the target specific assays. The primer and probe sequences are listed in table 2B and the locations are depicted in figure 1A.

QRT-PCR was performed in MicroAmp Optical 96-well Reaction Plates (ABI). A final reaction volume of 25 µl utilised the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase, with a final concentration of $MgSO_4$ adjusted to 5.6 mM, 20 units/reaction RNAseOUT (Invitrogen Corp., Carlsbad, California, USA), 50 mM ROX reference dye, and 500 ng of total RNA. Thermal cycling conditions for all Tyrosinase RT-PCR reactions were uniform across the set of assays. Reverse transcription incubation (54°C, 30 mi) was followed by an initial denaturing/RT inactivation incubation (94°C, 2 min) and directly linked to 42 cycles of PCR (15 seconds at 94°C, 45 s at 62°C, and 60 seconds at 68°C).

DNA AMPLIFICATION QUALITY: REAL-TIME PCR ASSAYS

Real-time qualitative PCR assays

Qualitative real-time PCR reactions were performed on the CFX96Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA). Resulting crossing threshold (Ct) data were analysed with the Bio-Rad CFX Manager Software V.1.6, utilising the second derivative maximum method.

PCR amplification and detection of qualitative PCR assays was performed in Multiplate PCR Plate Low 96 Well Clear (Bio-Rad). The final reaction volume of 25 µl contained Titanium Taq DNA Polymerase (Clontech Laboratories, Mountain View, California, USA), with the manufacturers standard buffer, 400 µM each dNTP and $1:10^4$ dilution SYBR-Green. PCR reactions were assembled with 400 nM of each assay specific amplification primer as described below. Each reaction was performed in duplicate, using 25 ng DNA from the respective specimen extractions for each reaction. Thermal cycling for detection of each of the nested targets employed an initial denaturing incubation (95°C, 3 min) followed by 42 cycles of PCR (20 s at 94°C, 80 s at 68°C).

BCOR DNA quality assay

BCL6 corepressor gene (BCOR, NM_001123383) sequence was the target for each of four DNA integrity assays. The BCOR PCR assays were designed to amplify nested fragments of different lengths from the same target DNA in separate, parallel, reactions (figure 2A). The fragment amplification of each assay used the same reverse primer (BCOR-R: 5′-ACTGACCCTGAAA CGTTAGTGATG-3′) paired with forward primers for each of the different BCOR amplicons (table 3).

CACNG4 DNA quality assay

Calcium channel, voltage-dependent, gamma subunit 4 gene (CACNG4, NM_014405) sequence was the target for each of three DNA integrity assays. The CACNG4 PCR assays were designed to amplify nested fragments of different lengths from

Table 3 PCR amplified DNA fragments and primers

Sequenced fragment assay ID	Amplicon length (bp)	Forward primer ID	Forward primer sequence (5′ to 3′):
BCOR-949	949	BCOR-949F	TCTACCTGCCGCCACCTC
BCOR-553	553	BCOR-553F	TGACAGTTAGCAGCGAGTTCC
BCOR-275	275	BCOR-275F	AGGGCTGGAAGTGGCTTAGT
BCOR-145	145	BCOR-145F	GCTGAATCAAATGATGGCAAAGTT
CACNG4-571	571	CACNG4-571R	CATCCTGTGTGACAAAAAGAGC
CACNG4-277	277	CACNG4-277R	CCTCGGTGGACCTTGAGC
CACNG4-117	117	CACNG4-117R	CACGGTCTCAGCCACAA

PCR amplified DNA fragments, as depicted in figure 2, used for PCR analysis of oligonucleotide fragmentation and sequencing quality analysis and the DNA oligonucleotides that are specific to PCR amplification and sequencing of each fragment. Unique oligonucleotide primers for sequencing BCOR gene fragments generate sequence in the forward direction. Unique oligonucleotide primers for the CACNG4 gene fragments generate sequence in the reverse direction.

the same target DNA in separate, parallel, reactions (figure 2B). The fragment amplification of each assay used the same forward primer (CACNG4-F: 5′-ATTTCCAGCAACACAGGTGACC-3′) paired with reverse primers for each of the different CACNG4 amplicons (table 3).

SANGER SEQUENCING ASSAYS

To evaluate the effect of the tissue preservation method on sequencing quality, amplified fragments from each patient specimen and for each fragmentation assay amplicon (each of four BCOR amplicons and three CACNG4 amplicons) were used for DNA sequencing analysis. Each of the 25 µl PCR reactions used for sequencing were diluted with 75 µl nuclease free water and transferred to individual wells of Multiscreen PCR µ96 Filter Plate (Millipore, Billerica, Massachusetts, USA). PCR amplified products were isolated by filtration under vacuum and resuspended with 20 µl nuclease free water.

The purified PCR products of each BCOR or CACNG4 reaction were bi-directionally sequenced using each amplification primer with ABI Big Dye V.3.1 Terminator Cycle Sequencing Kit. Dye terminator reactions were purified using Millipore Montage PCRu96 Filter Plate (Millipore, Billerica, Massachusetts, USA) and the sequences resolved by capillary electrophoresis using an ABI 3730xl DNA Analyser (Applied Biosystems, Foster City, California, USA).

RESULTS

Morphology and IHC comparison with different fixation methods

In order to investigate the conservation of morphological characteristics under PAXgene-fixation, H&E staining of PAXgene-fixed, paraffin-embedded (PAXPE) and FFPE specimens were evaluated in parallel. The morphology was well preserved in PAXPE specimens. No evident differences in comparison with FFPE tissues could be detected (figure 3).

For the purpose of comparing protein immunoreactivity between PAXPE and FFPE samples, IHC for 10 different antigens was performed using protocols previously established for FFPE. Markers of melanocytic differentiation such as S100, MITF, Tyrosinase and MelanA, as well as markers of tumour cell proliferation such as ki67, CCND1, p16 and p53 were investigated for staining intensity.

Using IHC protocols established for FFPE, staining as well as counterstaining was found to be generally stronger in FFPE compared with PAXPE tissues (figures 3 and 4, see online supplementary table S2). By evaluating the 12 patient samples for immunoreactivity, a significant difference between FFPE and PAXPE fixated tissues could be shown for b-Raf, MITF, CCND1, Tyrosinase, and MAGE-4 staining, which were significantly lower in PAXPE tissues. For S100 immunoreactivity, no difference between FFPE and PAXPE tissues could be detected. A moderate reduction in staining intensity was seen in PAXPE tissues compared with FFPE tissues for c-Kit, Ki67, p53, p16 and MelanA staining. However, these differences in intensity were not statistically significant (figure 4).

Whereas the signal intensity for Ki67 in PAXPE material could be increased by increasing the antibody concentration, staining intensity for p53 in PAXPE material could not be increased by increasing the antibody concentration (data not shown). Moreover, using anti-CCND1 and anti-p53 antibodies, different AR solutions were tested, but could not improve the staining signal (data not shown).

In order to facilitate nuclear staining, membrane permeability was increased using NP-40 in PAXPE tissues before adding MITF, p53, or CCND1 antibody. Staining increased proportionally with increasing membrane permeability in PAXPE tissues (figure 5).

In summary, IHC protocols established for FFPE tissues cannot be applied unmodified to PAXPE tissues since these seem to require different conditions.

NUCLEIC ACID QUALITY AS A FUNCTION OF FIXATION METHOD

To investigate the relative quality of nucleic acids from each tissue preservation method, RNA and DNA were extracted from each of the specimens. Total RNA and DNA were separately isolated from serial sections for each specimen with preservation specific methods using each manufacturer's recommended protocols. Extracted nucleic acids were quantified by absorbance and evaluated for impurities by 260/280 and 260/230 ratios for residual protein and chemical extraction contaminants respectively. Sample yield varied by cross-sectional tissue area and number of sections extracted. Since not all sections were slide mounted prior to extraction, yield per sectional area could not be calculated. Preservation method specific extraction methods did not show significant differences in sample purity as measured by 260/280 and 260/230 ratios (table 4). Performance effects based on co-purification of inhibitors that exhibit absorbance in these wavelengths was not observed at significant levels likely to exert negative effects on the nucleic acid performance in the assessment assays.

RNA QUALITY ASSESSMENT BY REVERSE TRANSCRIPTION PCR ASSAYS

To evaluate the RNA template quality as a function of the preservation method, qRT-PCR assays were designed to identify differences in the amplifiable copy number of the ABL1 kinase stable house-keeping gene, and a second transcript known to be variably expressed in malignant melanoma, Tyrosinase. RNA template quality was evaluated using both a 2-step cDNA synthesis followed by PCR, and a single tube 1-step method. QRT-PCR reactions were evaluated for each method using Real-Time PCR, and the resulting data analysed and quantified utilising the second derivative maximum method.

ABL1 kinase assay

ABL1 Kinase (ABL1, (NM_005157) mRNA was chosen as the target sequence for house-keeping gene expression. Three overlapping ABL1 amplicons were used for comparison of nucleic acid quality with 2-step RT-PCR chemistry. The assays were designed to amplify nested fragments, of increasing lengths from the same target mRNA in parallel reactions (figure 1B). Amplification primers for each of the different ABL1 RT-PCR assays are outlined in table 2.

Synthesis of ABL1 cDNA from total RNA was generated using the target specific primer ABL r2 primer for all of the assays. The ABL1 assays showed a strong trend toward higher integrity of template with the PAXPE samples over the FFPE samples. The level of expression varied between individual samples while the trend of greater amplification of the smallest fragments and less amplification of larger fragments correlated across all samples. For the ABL1 target amplification of the largest fragment (806 bp) failed to amplify for both fixation methods (figure 6).

Tyrosinase assay

Tyrosinase (TYR, NM_000372) mRNA expression levels were normalised to the 114 bp assay for the control gene ABL1 using

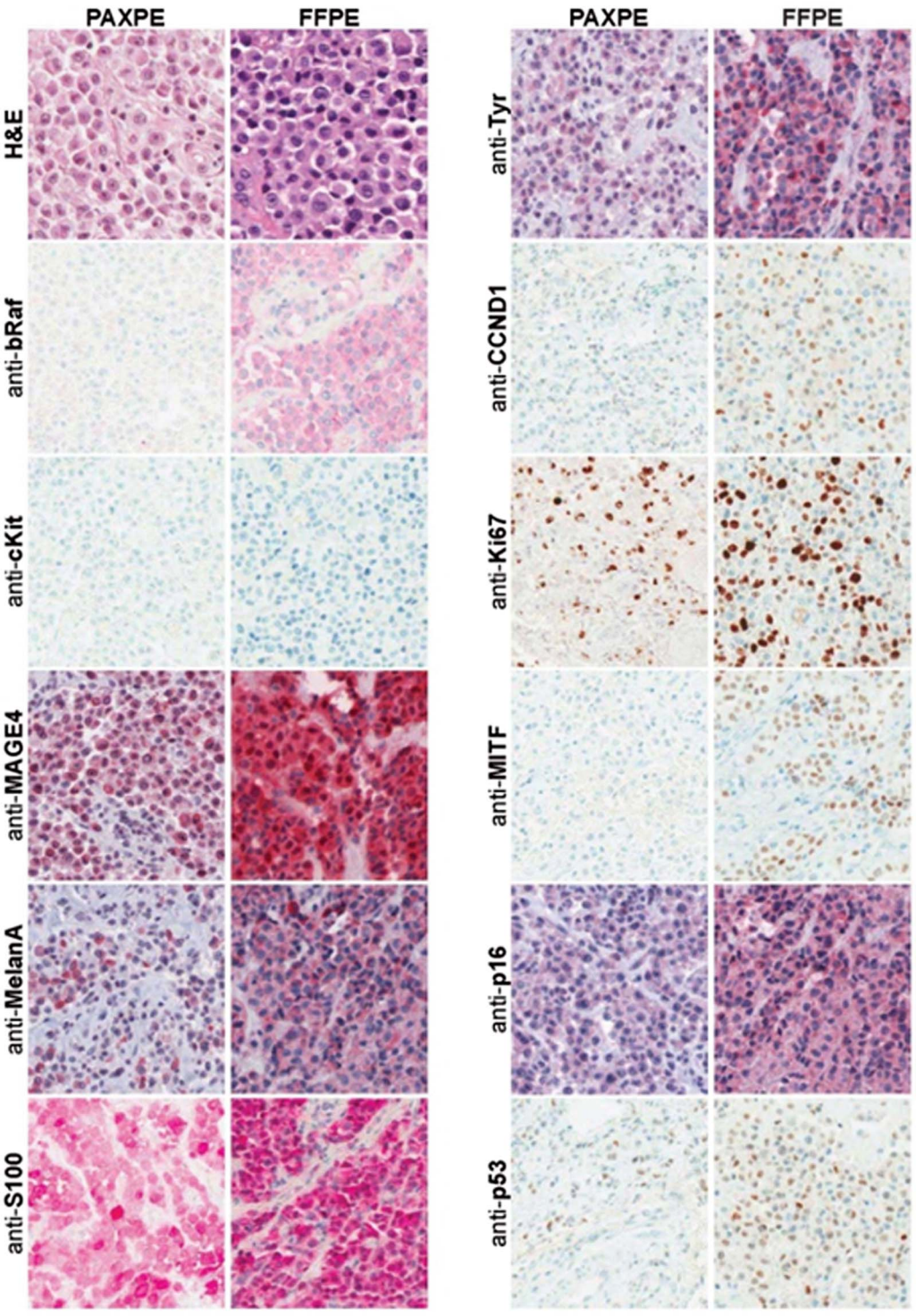

Figure 3 Comparison of H&E and immunohistochemistry staining in corresponding formalin-fixed, paraffin-embedded and PAXPE tissues. This figure is only reproduced in colour in the online version.

high quality in-tact RNA isolated from a melanoma cell line. To assess the quality of each preservation method, a series of increasing length one-step RT-PCR assays were designed to amplify nested fragments from the same target mRNA in parallel reactions. The quality of template preservation as it pertains to amplifiable length of RNA is reflected in the amplifiable target copy number as measured by the crossing threshold. To be able to compare the results between different length assays, the oligonucleotide primers and reaction conditions were optimised to attain similar qRT-PCR amplification efficiencies. The sequences are listed in table 2B and the locations are graphically depicted in figure 1A.

The quality between clinical isolates varies significantly which likely arises from the differences between the tumour material and processing time, precluding the ability to compare absolute values between clinical isolates. However, assays of increasing length evaluated within a specific tumour for each of the fixation methods shows a clear trend for greater template integrity in the PAXPE tissues relative to FFPE tissues (figure 7). In these assays additional intermediate size targets were generated

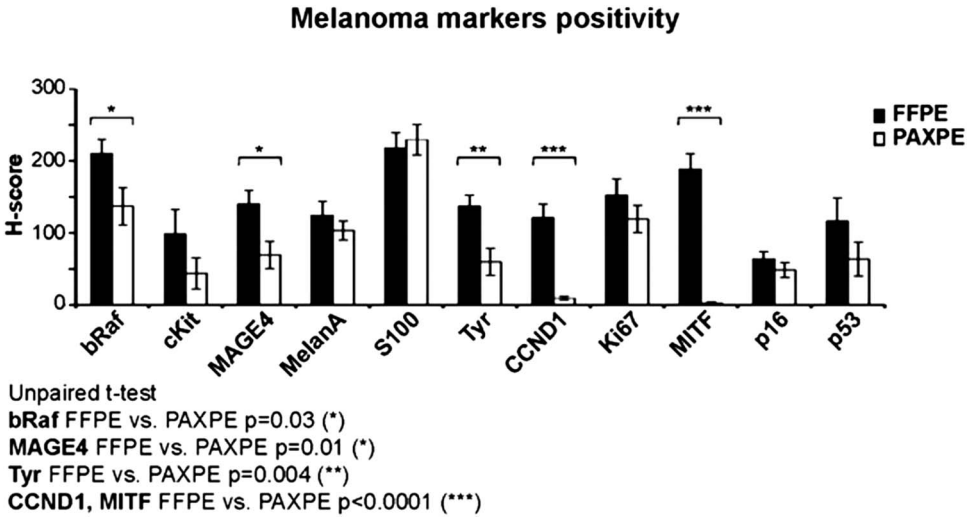

Figure 4 Comparison of staining intensity in corresponding formalin-fixed, paraffin-embedded and PAXPE tissues.

indicating available target template up to 712 bases in some samples. Although PAXPE fixed tissues exhibit significantly higher amounts of the larger amplicons, limitations of fragments larger than 500 bases are observed in most samples.

DNA QUALITY ASSESSMENT BY PCR AMPLIFICATION AND DIRECT SEQUENCING

Qualitative real-time PCR reactions were performed using non-specific double stranded DNA binding dye Sybr-green. Resulting data were analysed utilising the second derivative maximum method. Duplicate reactions using low amounts of template (25 ng) from each of the fixation methods were used to emphasise any differences in sample quality. Two target genes known not to vary in copy number in melanoma were chosen to avoid potential variation between clinical isolates.

DNA amplification assay

Assays were designed to amplify a series of nested fragment lengths, from the same target DNA in parallel reactions (figure 2). Similar to assays designed for examining the integrity of the RNA, amplification of each DNA target sequence utilised a common reverse primer paired with forward primers to generate a series of increasing size amplicons as depicted in table 3.

A dramatic loss of integrity was observed with the DNA amplification profiles for the BCOR and CACNG4 genes. The amplification of increasing size fragments are of measurably higher integrity in PAXPE tissues compared with FFPE. Both target genes exhibit the same trend across all samples. Specimens 11 and 12 were of notably less integrity for both paxgene-fixed, paraffin-embedded (PAXPE) and FFPE specimens, presumably a result of pre-fixation handling (figure 8).

Sequencing analysis

To evaluate the effect of the tissue preservation method on sequencing quality, amplified fragments from each patient specimen, for each fragmentation assay amplicon (each of four BCOR amplicons and three CACNG4 amplicons) were used as template for DNA sequencing analysis.

The purified PCR products of each BCOR or CACNG4 reaction were sequenced in both directions and analysed for

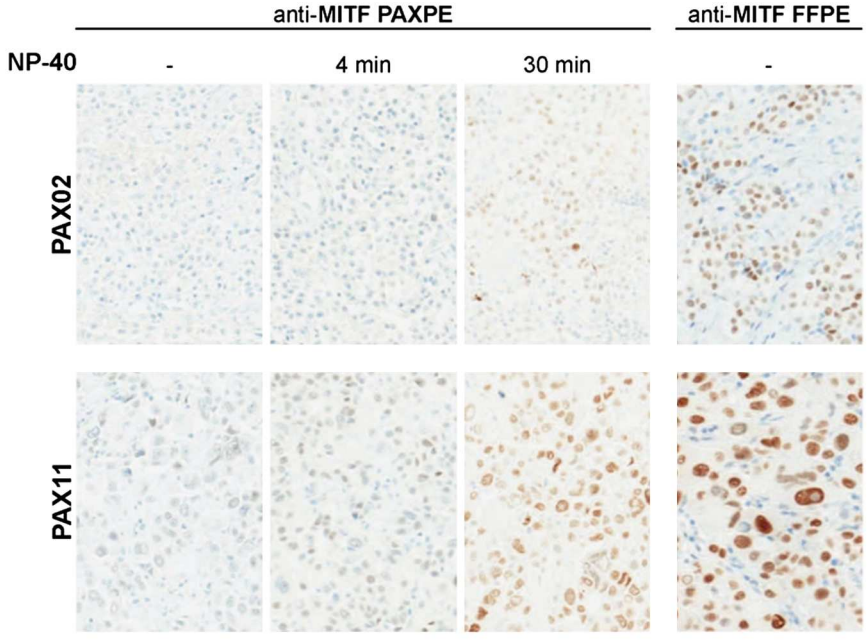

Figure 5 Increase of nuclear staining under NP-40 treatment of PAXPE tissues in comparison with the corresponding formalin-fixed, paraffin-embedded tissue. This figure is only reproduced in colour in the online version.

Table 4 Spectrophotometric measurement of extracted nucleic acids

			RNA Extractions				DNA Extractions			
Patient (specimen) ID:	Tissue fixation	Extraction method	A_{260}	A_{280}	$A_{260/280}$	$A_{260/230}$	A_{260}	A_{280}	$A_{260/280}$	$A_{260/230}$
P1	PaxGene	PreAnalytix	1.67	0.82	2.02	1.58	2.23	1.17	1.90	2.51
	Formalin	MoBio	0.47	0.23	2.00	1.89	0.23	0.11	2.06	2.06
	Frozen	Trizol	0.68	0.35	1.94	1.20				
P2	PaxGene	PreAnalytix	3.19	1.57	2.03	1.96	3.27	1.62	2.02	2.38
	Formalin	MoBio	1.59	0.77	2.06	2.03	0.12	0.04	3.23	1.55
	Frozen	Trizol	2.41	1.20	2.00	1.86				
P3	PaxGene	PreAnalytix	2.64	1.32	2.00	1.50	6.16	3.18	1.94	2.40
	Formalin	MoBio	10.13	4.92	2.06	2.12	14.71	7.38	1.99	2.26
	Frozen	Trizol	5.87	3.02	1.94	1.33				
P4	PaxGene	PreAnalytix	0.93	0.46	2.02	0.56	4.77	2.44	1.96	2.30
	Formalin	MoBio	4.65	2.37	1.96	1.78	9.13	4.67	1.96	2.25
	Frozen	Trizol	7.65	4.14	1.85	1.07				
P5	PaxGene	PreAnalytix	0.72	0.40	1.81	0.56	4.41	2.90	1.52	0.93
	Formalin	MoBio	2.58	1.34	1.93	1.55	2.31	1.23	1.88	1.76
	Frozen	Trizol	1.64	0.95	1.73	0.69				
P6	PaxGene	PreAnalytix	0.51	0.28	1.81	0.36	1.57	0.82	1.93	2.35
	Formalin	MoBio	2.00	1.04	1.93	0.89	1.43	0.71	2.01	2.14
	Frozen	Trizol	1.73	0.96	1.79	0.81				
P7	PaxGene	PreAnalytix	0.69	0.35	1.96	1.39	3.95	1.97	2.01	2.24
	Formalin	MoBio	7.64	3.92	1.95	1.91	22.32	11.15	2.00	2.22
	Frozen	Trizol	0.90	0.52	1.74	0.63				
P8	PaxGene	PreAnalytix	1.70	0.82	2.06	1.95	3.75	1.86	2.02	2.29
	Formalin	MoBio	5.82	2.98	1.95	1.58	13.73	6.89	1.99	2.25
	Frozen	Trizol	3.19	1.67	1.91	1.03				
P9	PaxGene	PreAnalytix	4.52	2.18	2.07	0.69	2.80	1.38	2.04	0.41
	Formalin	MoBio	7.89	3.97	1.99	1.76	12.65	6.27	2.02	2.13
	Frozen	Trizol	6.59	4.04	1.63	0.82				
P10	PaxGene	PreAnalytix	2.14	1.05	2.04	0.85	5.74	2.89	1.99	2.32
	Formalin	MoBio	3.69	1.86	1.99	0.85	11.35	5.70	1.99	2.23
	Frozen	Trizol	3.35	1.70	1.98	1.37				
P11	PaxGene	PreAnalytix	4.94	2.41	2.05	0.86	15.62	7.90	1.98	2.22
	Formalin	MoBio	3.97	1.99	1.99	1.68	2.34	1.20	1.96	0.48
	Frozen	Trizol	2.79	1.42	1.97	1.56				
P12	PaxGene	PreAnalytix	0.93	0.47	1.98	0.62	3.79	1.83	2.07	2.25
	Formalin	MoBio	3.16	1.62	1.96	1.97	6.87	3.44	1.99	2.27
	Frozen	Trizol	19.24	12.13	1.59	0.84				

DNA and RNA were extracted from different portions of the same specimens from each of the preservation methods. Limited size of specimens did not allow for extraction of DNA from frozen specimens. Absorbance of each extracted sample was measured by spectrophotometer (Nanodrop ND1000) and nucleic acid sample purity assessed by ratios of absorbance 260 nm/280 nm (A260/280) and absorbance 260 nm/230 nm (A260/230). A260/280 of ~1.8, or greater, is generally accepted as 'pure' for DNA; A260/280 of ~2.0 is generally accepted as 'pure' for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein or other contaminants that absorb strongly at or near 280 nm. If the A260/280 value is appreciably lower than ~2.0–2.2, it may indicate the presence of residual organic contaminants which absorb at 230 nm.

sequencing quality and read length. Since PCR was allowed to cycle to plateau the differences in amplification resulting from reduced copy number were compensated by extensive amplification. No significant difference in read length or sequence quality was observed to correlate with amplicon length (data not shown).

DISCUSSION

Formalin fixation and paraffin embedding has been the standard routine procedure for preservation of human specimens for decades. Stored tissues in pathology department archives present an important resource for future detailed clinical and molecular analysis of diseases. Formalin enables cost-effective long-term storage of human samples and preserves detailed cellular morphology. However, formalin fixation leads to cross-linking of proteins as well as fragmentation of nucleic acids, impairing the quality of extracted proteins as well as RNA and DNA.[5 13 14] The formalin-free PAXgene tissue system was designed to improve the quality of molecular analysis without diminishing the quality of histopathological analysis.

Concerning morphological analysis, morphology was well preserved in PAXPE samples. H&E staining revealed no differences between FFPE and PAXPE tissues concerning tissue architecture, cellular and nuclear morphology, apart from a slightly stronger staining in FFPE tissues. Higher eosinophilia was not observed in our samples, in contrast to the study reported by Kap *et al*.[15] This difference might be due to the different tissue types used. Difficulties in tissue handling as described for other formalin-free, alcohol and acid based fixatives such as tissue hardening or softening, tissue shrinkage, pigment deposition, and lysis of erythrocytes were not observed.[6] The good morphological results of PAXPE in comparison with other studies conducted on formalin-free fixatives may be attributed in part to the small size of tissue samples (4×10×10 mm as suggested by the manufacturer), the resulting short fixation time of 2–4 h, and the tissue type used (melanoma samples from skin and lymph nodes).

Immunohistochemical analysis revealed a generally lower counterstaining in PAXPE tissues compared with FFPE material. Depending on immunohistochemical marker tested, significant

Relative RNA Amplification Values ($2^{-\Delta Ct}$)

Patient ID	Tissue Fixation	ABL1 Amplicon Length 114 bp	398 bp	806 bp
P1	PaxPE	0.0165	0.0076	0
	FFPE	0.0041	0	0
P2	PaxPE	0.2952	0.176	0
	FFPE	0.0054	0.0009	0
P3	PaxPE	0.1934	0.1181	0
	FFPE	0.0012	0	0
P4	PaxPE	0	0	0
	FFPE	0.0008	0	0
P5	PaxPE	0.0038	0	0
	FFPE	0.0006	0	0
P6	PaxPE	0.0041	0	0
	FFPE	0.0019	0.0022	0
P7	PaxPE	0.0602	0.0605	0
	FFPE	0.0051	0	0
P8	PaxPE	0.1216	0.1121	0
	FFPE	0.0036	0	0
P9	PaxPE	0.4813	0.5046	0
	FFPE	0.001	0	0
P10	PaxPE	0.1358	0.0804	0
	FFPE	0.7145	0.7747	0.0002
P11	PaxPE	0.0016	0	0
	FFPE	0.0027	0	0
P12	PaxPE	0.0027	0	0
	FFPE	0.0103	0.0017	0

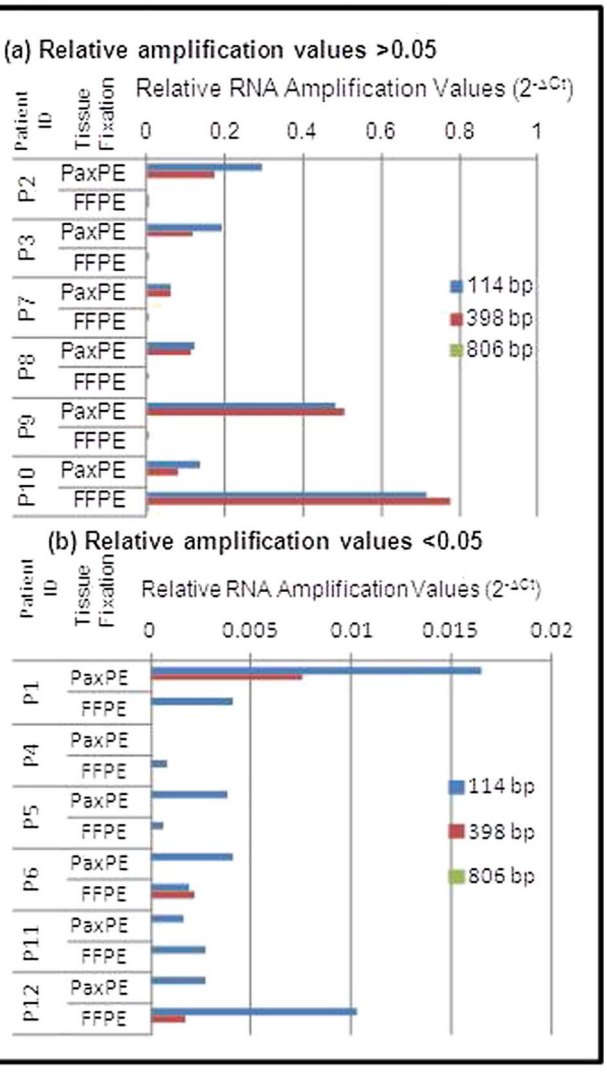

Figure 6 RNA Amplification Quality Assay—ABL Tyrosine Kinase Assay. The figure shows the calculated data and graphed results. The graphically depicted data has been divided into two separate graphs with different scales for the relative amplification values to make all the lower expression values more visible. Data is assigned to the respective graphs according to the maximum amplification value within a patient sample set. Amplification quality is calculated as a 2^{-DCt} value ($DC_t=C_{t\ (experimental\ RNA)}—C_{t\ (control\ RNA)}$) to allow comparison of amplification quality scores on a linear scale. The amplification of each length of ABL1 is measured relative to amplification of the same ABL1 amplicon from control RNA extracted from cultured cells. Amplicons with 2^{-DCt} values close to 1.0 indicate that the target, from the respective RNA sample, is close to being fully in-tact. 2^{-DCt} approaching zero for demonstrate greater degradation of the target in the experimental RNA sample. This figure is only reproduced in colour in the online version.

differences in staining intensity could be detected. 5 out of 11 markers showed significantly lower overall staining with PAXPE tissues in comparison with FFPE. Increasing antibody concentration could improve staining intensity for some markers in PAXPE tissues but not for all. Pre-treatment with different AR methods, in order to unmask antigen epitopes by enzymatic predigestion, as well as noAR, did not improve staining quality of PAXPE tissues. However, increasing membrane permeability through adding a detergent did proportionally increase staining intensity of nuclear markers in PAXPE samples. The present study shows that immunohistochemical protocols developed for FFPE tissues require extensive optimisation and validation before being used for PAXPE tissues. A recently published study demonstrated successful extraction of non-degraded and immunoreactive protein for downstream applications such as western blotting reverse-phase protein microarrays.[5] Results of the immunohistochemical analysis were recently reported by Kap *et al*.[15] The authors report, only a few IHC antibodies exhibited lower levels of immune reactivity which could be overcome by omittingAR, adjusting antibody concentration, or using another clone. However, the authors only present single case examples and do not provide validated scoring results or statistics.

Concerning other studies performed with formalin-free, alcohol and acid based fixatives, a reduction of staining intensity and quality was observed, depending on the markers used.[6]

For RNA analysis, the amplification of different mRNA amplicons showed significant differences between PAXPE and FFPE samples. FF, which presents the intact template, was significantly better. This data is consistent with previous reports of Ergin *et al* and Viertler *et al*. The authors report amplification of longer amplicons from PAXPE and FF samples, whereas for FFPE only shorter amplicons could be amplified.[5 16]

Relative RNAAmplification Values($2^{-\Delta Ct}$)

Patient ID	Tissue Fixation	Tyrosinase Amplicon Length 108 bp	338 bp	589 bp	712 bp
P1	PaxPE	0.0665	0.023	0.0003	0.0004
	FFPE	0.0928	0.0263	0.0003	0
P2	PaxPE	0.1426	0.2698	0.0272	0.0145
	FFPE	0.1066	0.0235	0.0001	0.0002
P3	PaxPE	0.7371	0.356	0.4061	0.3276
	FFPE	0.4506	0.0634	0	0
P4	PaxPE	0.5471	0.1638	0.1037	0.083
	FFPE	0.5176	0.0556	0	0
P5	PaxPE	0.2349	0.4175	0.086	0.086
	FFPE	0.2253	0.5396	0.0008	0
P6	PaxPE	0.507	0.4538	0.0053	0
	FFPE	0.1029	0.0058	0	0
P7	PaxPE	0.2045	0.3463	0.0526	0.1387
	FFPE	0.483	0.1416	0	0.0162
P8	PaxPE	0.1088	0.1349	0.2365	0.2253
	FFPE	0.507	0.0152	0	0
P9	PaxPE	0.8123	0.5625	0.0209	0.0216
	FFPE	0.1856	0.732	0.0073	0.0019
P10	PaxPE	0.1456	0.3035	0.1207	0.1134
	FFPE	0.1731	0.0254	0.0001	0
P10	PaxPE	0.1426	0.1312	0	0
	FFPE	0.5946	0.0052	0	0
P11	PaxPE	0.1426	0.1312	0	0
	FFPE	0.5946	0.0052	0	0
P12	PaxPE	0.3511	0.029	0	0
	FFPE	0	0	0	0

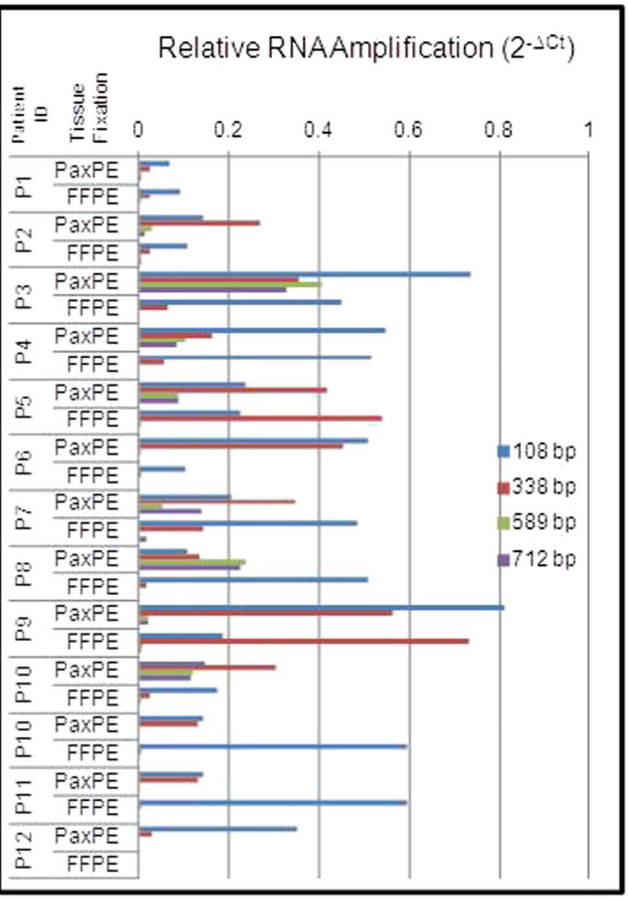

Figure 7 RNA Amplification Quality—Tyrosinase Assay. The table and graph show the same data. The amplification of each length of TYR is measured relative to amplification of a control ABL1 fragment (114 bp) from the same RNA cell line. Amplification quality is calculated as a 2^{-DCt} value ($DC_t=C_{t\ (TYR\ amplicon)}—C_{t\ (ABL1\ control)}$) to allow for comparing amplification quality scores on a linear scale. Amplicons with 2^{-DCt} values close to 1.0 show that the target, from the respective RNA sample, is close to being fully in-tact. 2^{-DCt} approaching zero for demonstrate greater degradation of the target in the experimental RNA sample. This figure is only reproduced in colour in the online version.

For DNA analysis, sequencing and mutational analysis are comparable for all the different fixation methods, while the level of DNA fragmentation seems to be lower in PAXPE compared with FFPE tissues. The extensive amplification of target template during the PCR step of sequencing masks the differences in copy number. These differences are however observed in the comparative analysis of DNA quality and target fragment content using real-time PCR with Sybr-green(TM). Fixation specific extraction methods further complicate the observed differences in template quality. Analysis of both RNA and DNA derived from PAXgene tissue suggest that it may be useful for assays that span greater fragment length such as translocation events. Larger amplifiable fragments could also reduce the number of individual reactions required to span larger exons or for nextgen sequencing of mutations to determine whether clinically relevant mutations are found in cis or trans to one another.

Although the PAXgene fixative displays improved preservation of nucleic acids, the switch from formalin to PAXgene fixation would require a re-evaluation of markers and staining procedures because protocols as well as information regarding sensitivity and specificity have been obtained using formalin-fixed samples. Furthermore, preservation of morphological as well as molecular features after prolonged storage (eg, 10 years) of PAXPE tissues needs still to be evaluated.

The small sample size of 4×10×10 mm for PAXgene fixation is a major limit to application in routine pathology, since most surgical specimens are of bigger sizes. The associated costs of this fixation system are a multiple compared with inexpensive formalin, and need to be taken into account, as well as the necessary logistics, such as the recommended storage of PAXPE blocks at 4°C compared with room temperature for FFPE, when selecting a fixative for routine pathology. However, when using small sample sizes, fixation times of only 2–4 h using PAX gene compared with 24 h using formalin can facilitate and accelerate workflow in laboratories. Furthermore, carcinogenic effects of formalin in laboratory personnel could be more easily avoided by creating a formalin-free environment, but with the addition of handling toxic and highly inflammable methanol-containing substances.

Our data demonstrate that PAXPE fixation offers some key advantages in nucleic acid detection that may be useful for specific disease linked markers that require longer amplicon analysis. Currently, for routine pathology laboratory work, we recommend continuing to use FFPE fixation.

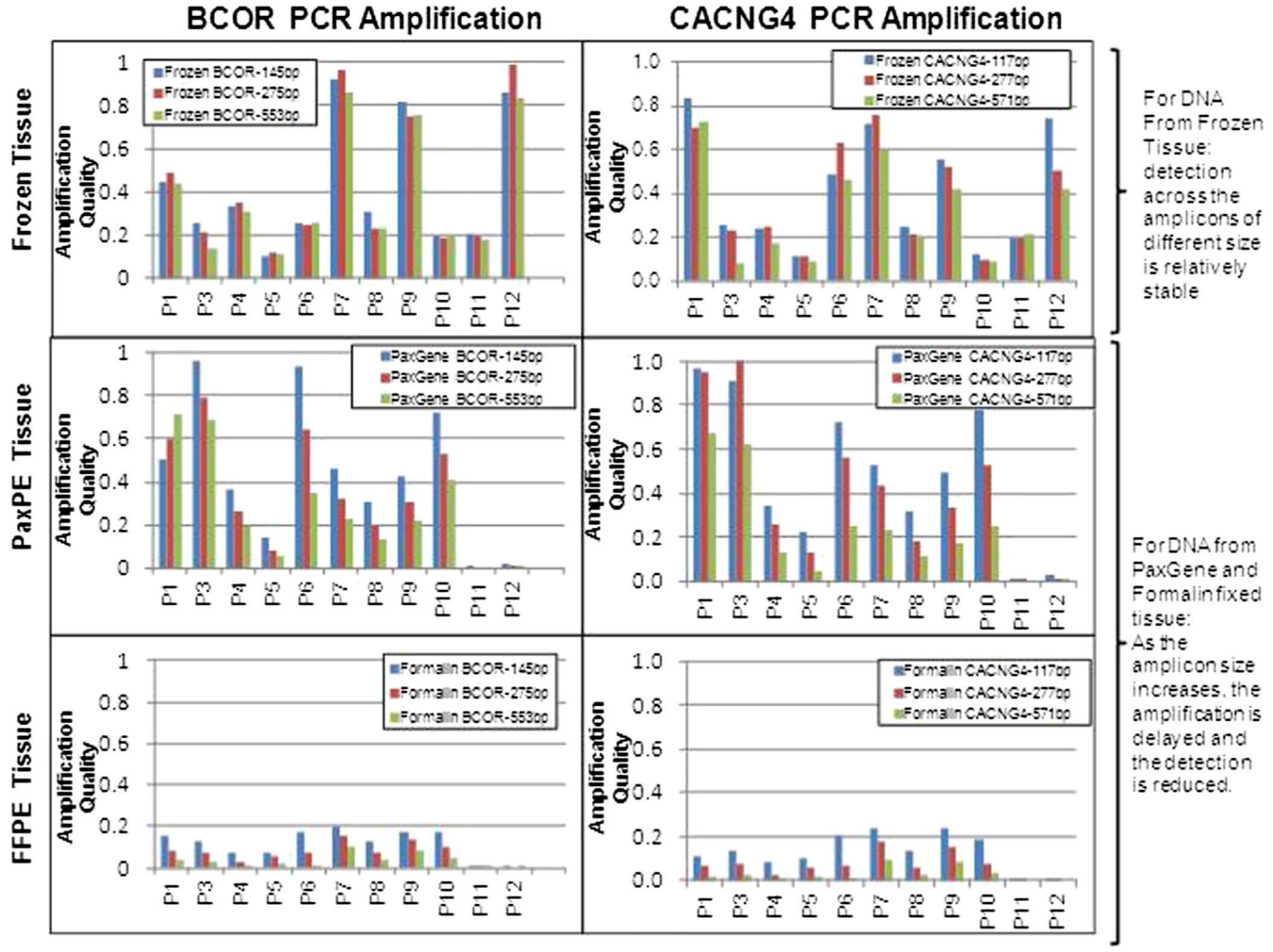

Figure 8 DNA Amplification Quality Real-Time PCR Assays. Amplification quality of DNA from each patient is compared between different tissue preservation methods. Amplification quality of patient samples is shown for two separate gene sequences, present on different chromosomes, and represented relative a control sample of relatively highly in-tact genomic DNA (DNA extracted from cell lines by Trizol method). The relative amplification quality is plotted as a 2^{-DDCt} value calculated from the Ct value of each amplicon as it is amplified from each experimental sample, using the Ct value of the respective amplicons amplified from the control DNA. Amplification quality values approaching=1 have little DNA fragmentation. Amplification quality scores approaching zero indicate greater fragmentation of DNA and lower amplification quality. This figure is only reproduced in colour in the online version.

Take home messages

- In PAXPE samples, morphology is well preserved but immunohistochemistry requires re-evaluation of markers and staining procedures.
- PAXPE samples provide greater template integrity of mRNA amplicons than formalin-fixed, paraffin-embedded samples.
- DNA fragmentation seems to be lower in PAXPE samples compared with formalin-fixed, paraffin-embedded samples.
- The authors would not suggest substituting formalin fixation with PAXgene fixation in a routine pathology laboratory.

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REFERENCES

1 Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem* 1991;39:741–8.
2 Lehmann U, Kreipe H. Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. *Methods* 2001;25:409–18.
3 Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am J Pathol* 2002;161:1961–71.
4 Masuda N, Ohnishi T, Kawamoto S, *et al*. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res* 1999;27:4436–43.
5 Ergin B, Meding S, Langer R, *et al*. Proteomic analysis of PAXgene-fixed tissues. *J Proteome Res* 2010;9:5188–96.
6 Moelans CB, ter Hoeve N, van Ginkel JW, *et al*. Formaldehyde substitute fixatives. Analysis of macroscopy, morphologic analysis, and immunohistochemical analysis. *Am J Clin Pathol* 2011;136:548–56.

7 Moelans CB, Oostenrijk D, Moons MJ, *et al*. Formaldehyde substitute fixatives: effects on nucleic acid preservation. *J Clin Pathol* 2011;64:960–7.
8 Vincek V, Nassiri M, Nadji M, *et al*. A tissue fixative that protects macromolecules (DNA, RNA, and protein) and histomorphology in clinical samples. *Lab Invest* 2003;83:1427–35.
9 Delfour C, Roger P, Bret C, *et al*. RCL2, a new fixative, preserves morphology and nucleic acid integrity in paraffin-embedded breast carcinoma and microdissected breast tumor cells. *J Mol Diagn* 2006;8:157–69.
10 Uneyama C, Shibutani M, Masutomi N, *et al*. Methacarn fixation for genomic DNA analysis in microdissected, paraffin-embedded tissue specimens. *J Histochem Cytochem* 2002;50:1237–45.
11 Morote J, Fernandez S, Alana L, *et al*. PTOV1 expression predicts prostate cancer in men with isolated high-grade prostatic intraepithelial neoplasia in needle biopsy. *Clin Cancer Res* 2008;14:2617–22.
12 Kinsel LB, Szabo E, Greene GL Jr, *et al*. Immunocytochemical analysis of estrogen receptors as a predictor of prognosis in breast cancer patients: comparison with quantitative biochemical methods. *Cancer Res* 1989;49:1052–6.
13 Stanta G, Mucelli SP, Petrera F, *et al*. A novel fixative improves opportunities of nucleic acids and proteomic analysis in human archive's tissues. *Diagn Mol Pathol* 2006;15:115–23.
14 Puchtler H, Meloan SN. On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions. *Histochemistry* 1985;82:201–4.
15 Kap M, Smedts F, Oosterhuis W, *et al*. Histological assessment of PAXgene tissue fixation and stabilization reagents. *PLoS One* 2011;6:e27704.
16 Viertler C, Groelz D, Gundisch S, *et al*. A new technology for stabilization of biomolecules in tissues for combined histological and molecular analyses. *J Mol Diagn* 2012(5):458–66.

Will PAXgene substitute formalin? A morphological and molecular comparative study using a new fixative system

Benedetta Belloni, Chiara Lambertini, Paolo Nuciforo, et al.

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